
Characterisation of *P. falciparum* antigenic determinants isolated from a genomic expression library by differential antibody screening

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ABSTRACT

A genomic expression library of *P.falciparum* has been differentially screened with a number of immune sera. The response of 9 clones to the various sera is presented, together with the DNA sequence encoding the epitopes. All but one clone are extremely A+T rich and unlike the other *P.falciparum* epitopes described, are not composed of amino acid repeats. One clone, which responds specifically with a protective serum, has been analysed in detail. The epitope is carried on a 160kd antigen which is transcribed from a single gene to give a protein expressed in all of the erythrocytic forms. DNA sequence of this clone reveals it to have more than one open reading frame, only one of which is transcribed in the blood stages. The possible significance of the other open readings frames is discussed.

INTRODUCTION

The parasite *Plasmodium falciparum* has a complex life-cycle which includes replication in the mosquito vector as well as in man, where it causes malaria. All studies to date indicate that it synthesizes many proteins and presents a large number of antigens to the host's immune-system. Although there are several good candidates it is not yet known which antigen(s) are important in the development of protective immunity (1). In an attempt to define those important antigens, most workers have focussed on a particular protein and by the use of specific sera, have been able to isolate the corresponding gene from expression libraries (2, 3, 4, 5, 6). Another approach, exemplified by Kemp and his co workers (7, 8) is to isolate a large number of antigenic determinants from a cDNA expression library using an immune poly-specific serum. Analysis of, and comparison between these determinants is then possible.

Here, we describe the characterisation of a number of clones encoding antigenic determinants, isolated from a genomic expression library. A genomic expression library was constructed, because unlike a cDNA library all unique genes are equally well represented independant of the level of transcription or the developmental stage in which they are expressed. We have previously reported the characterisation of one of these clones, pPF11-1 (9) and now we present the analysis of eight others identified in a number of differential antibody screening.

MATERIALS AND METHODS

Construction and Screening of genomic library

A preliminary description of the construction has been reported in (9). A detailed account of the immunoenzymatic screening method appears in (10). In brief, *P.falciparum* genomic DNA was digested with DNaseI and size fractionated on a 6% polyacrylimide gel. DNA of size 50-1000 bp was eluted, GC tailed and cloned into the *Pst* I site of vector PUK270. 6×10^4 transformants per ug of annealed vector were obtained.

Immunoprecipitations

Parasites were labelled according to the protocol in ref.11 and the immunoprecipitations with monkey sera were done essentially as described in ref.11. 3000,000 cpm of 35 -S methionine labelled asynchronous parasites were extracted with Triton and the immunoprecipitations performed with 5ul of each of the two monkey sera. They were analysed on a 10% SDS-PAGE.

Western blotting

Immunodetection of the purified fusion protein with anti-*P.falciparum* antibodies. pPF2L was grown overnight at 37°C in L Broth supplemented with 25 ug/ml ampicillin and 1mM isopropyl-B-D-thio-galactoside (IPTG). The fusion protein was purified according to (12). 1ug. of protein was loaded on a 7.5% SDS-PAGE and after electrophoresis transferred to nitrocellulose. Immunodetection was made according to (13). A pool of immune human sera against *P.falciparum* where anti *E.coli* antibodies had been carefully removed, was used. Immunocomplexes were revealed by iodinated protein A.

Specificity of antibodies to pPF2L antigen

Antibodies were raised in Balb/C mice by injecting 10 ug of the purified pPF2L protein in PBS/0.5%SDS emulsified in Freund's complete adjuvant on day 0, and in Freund's incomplete adjuvant 1 month and 3 months later. Bleeding was done 1 week after the last injection. Immunoprecipitations were performed as above. 10 ul. of both the pre-immune and anti-pPF2L sera were used.

Indirect Immunofluorescence (IFA)

Thin smears of asynchronous culture parasites were fixed with acetone and then incubated with a 1/20 dilution of mouse antiserum raised against purified SDS-denatured pPF2L protein, as described in (9). The immunocomplexes were then revealed by fluorescein conjugated anti-mouse IgG rabbit antibodies.

Southerns and Northern

The protocols used have been previously described (14). *P.falciparum* (FUP strain) DNA (2 ug/track) was cut with *Eco*R1, *Hind*III and *Sau*3A, under conditions recommended by the manufacturer. The restricted DNA was fractionated, together with pBR322 size markers, by 0.8% agarose gel electrophoresis, before transfer to nitrocellulose. The transfer was then hybridized with the *P.falciparum* DNA insert of pPF2L. The hybridisation solution included dextran sulphate at 5% concentration. The filter was washed in 2X SSC at 37°C for 4 hours, with 8 changes, before exposure to film. 2ug. of *P.falciparum* blood stage poly A+ RNA was fractionated on a 1% agarose/formaldehyde gel, transferred to nitrocellulose and then subjected to the same regime as the Southern transfer.

DNA sequencing

The sequences presented in Fig 1 were derived by the method of Maxam and Gilbert (15). The purified insert of pPF2L was restricted with various endonucleases and then blunt end ligated into the *Sma*I site of the phage M13mp8 DNA. The nucleotide sequence was then determined by standard procedures (16). The strain used, TG-1, is a restriction deficient derivative of JM101 (17) and was kindly provided by T. Gibson (Cambridge).

RESULTSDifferential immuno-screening of the genomic expression library

A preliminary description of the construction of the

Table 1 Properties of nine *P. falciparum* antigen producing clones

name of pPf clone	A. Properties of the Inserts		B. Reaction with various antisera				
	size (bp)	dA + dT content (%)	rabbit (T ₅)	human (Africa)	human (South America)	monkey (422) (protective)	monkey (017) (nonprotective)
41-2	216	77	+	+	+++	+++	+++
35-75	132	82	+	+	-	+	+
3-12	75	77	+	+	-	+	-
22-1	93	77	+	+	+++	+++	+++
20-3	33	88	+	-	+++	+++	+++
4-1	93	89	+	+	+	-	-
56-52	108	42	+	+++	+	++	+
11-1	620	65	+	+++	-	-	-
2L	689	81	+	++	++	+	-

Table 1 : Properties of nine *P. falciparum* antigen expressing clones. Table 1.A shows the size (without tails) and the dA/dT content of the inserts of nine *P.falciparum* clones. Immunological specificity (Table 1.B) of the nine *P. falciparum* antigens were tested with the following antisera: rabbit antiserum raised against Triton extracted *P.falciparum* asexual blood stage proteins, a mixture of sera from immune African humans, an immune human serum from South America (Columbia), a protective and a nonprotective monkey sera. Rabbit serum reactivity against the nine clones was tested by the immunoenzymatic method (9). All other sera were tested by *in situ* colony immunoassay (7). The latter method allows a graduate reaction signal determination : + = weak, ++ = strong, +++ = very strong.

genomic expression library appears in (9) and its screening in (10). In brief, small (50bp-1000bp) DNase I generated fragments were cloned into the 5' end of the B-galactosidase lacZ gene. 10,000 actively expressing clones were then selected on lactose minimal plates. These were subjected to a number of screenings with different immune sera. First, a rabbit serum (T5), raised to parasite blood stage proteins, detected 100 clones encoding antigenic determinants of which 36 remained reproducibly positive. Second, a pool of sera from Africans immune to malaria identified 15 clones, 9 of which were also positive with the rabbit T5 serum. These nine clones are described here. They were further analysed with an immune human serum from South America and with a protective (422) and non-protective (017) serum from Squirrel monkeys (11). A protective serum is defined as being able to confer protection against *P.falciparum* to a naïve monkey on passive transfer. The results of the immunological properties of the nine clones are summarised in Table 1b.

Two points can be deduced from Table 1b. The fact that all these clones show immunoreactivity with the rabbit antiserum (T5) raised to erythrocytic forms of the parasite, suggests that the determinants detected here are expressed at least during the erythrocytic cycle. Apart from T5, each antiserum shows a specific pattern of reaction with the panel of clones analysed and inversely most of the clones have a specific combination of reactivity with the sera used. This encourages us to believe that the probably most of the clones encode genuine antigenic determinants.

Nucleotide sequence encoding the antigenic determinants

The analysis and sequence of pPF11-1 has been reported (9). Clone pPF2L is analysed in detail below. Fig 1 shows the nucleotide sequence of the other clones. The library was constructed in such a way that the antigenic determinants are produced fused to active B-galactosidase. Furthermore, each clone gives a positive immunoenzymatic reaction, which indicates that the active fusion proteins are bound to the *P.falciparum* antibodies (10,23). The nucleotide sequence was determined and the inferred amino-acid sequence presented is that in-frame with the lac Z gene. The cloned insert size varies considerably, ranging from 689bp to 33bp (Table 1a) with a bias towards the smaller inserts. The A+T content of nearly all the clones is high (80%), only one clone pPF56-52 has a low A+T content (42%).

For all clones except for pPF35-75 and pPF4-1, the open reading frame runs throughout the entire insert. These two clones, like pPF2L, appear to express B-galactosidase fusions as a result from a reinitiation of translation. Although they possess other reading frames, the one in-frame with B-galactosidase is known to encode an antigenic determinant, as the clones were recognised by an immuno-enzymatic assay (23). Whether the other frames are expressed is unknown at this time. All the clones encode amino-acids that are highly charged. We don't think this has resulted in an artifactual response, since the clones react differently to the various sera. The significance of these epitopes is discussed.

We now present a more detailed analysis of clone pPF2L. It was chosen as it has the largest insert (689bp) and because it responds to both the African and South American human sera. Furthermore, it shows a weak but specific response to the protec-

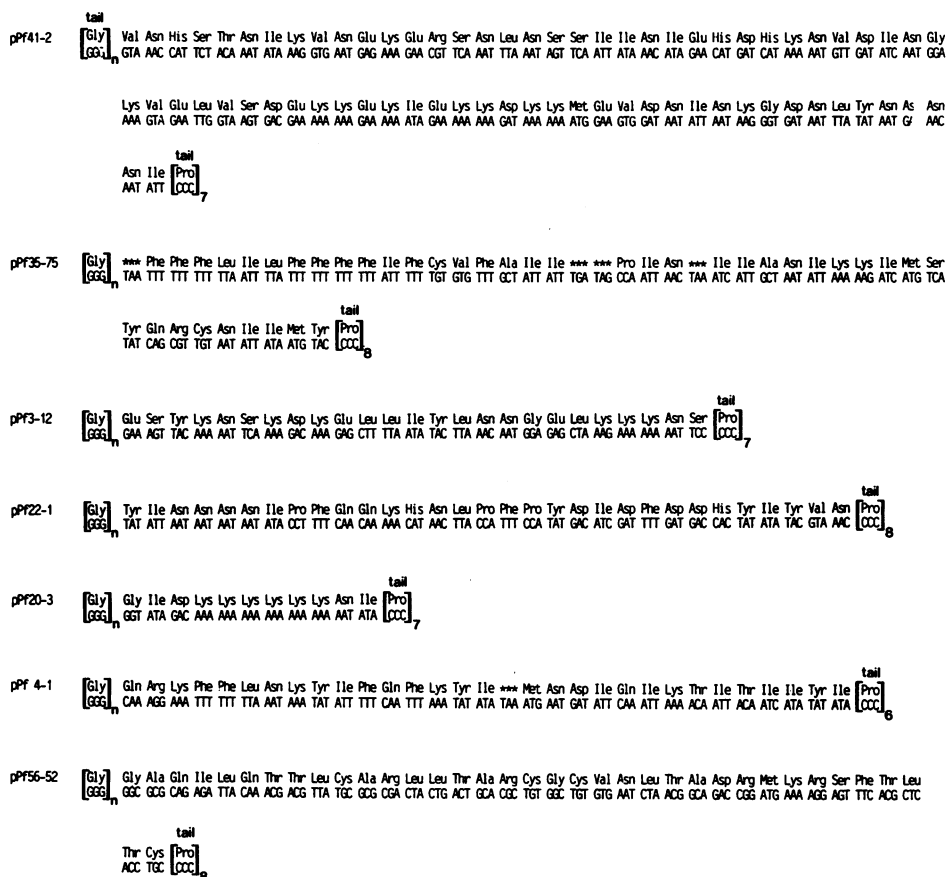


FIGURE 1. DNA sequence of genomic clones expressing antigenic determinants of *P.falciparum*. DNA sequence was determined according to Maxam & Gilbert (15). Recombinant plasmids were isolated as described elsewhere (22). The polylinker of pUK270 allowed direct chemical sequencing from both sides of the insert (23). The reading frames are shown to be in phase with the lac Z gene.

tive monkey serum and its recognition therefore may play a role in the development of protective immunity.

Identification of the pPF2L antigen

The fusion protein produced by pPF2L was purified by affinity chromatography (12) and shown to react with human sera (Fig 2b). Two bands can be seen which result from the B-galactosidase fusion and a breakdown product, which is routinely produced on purification. This purified chimera was then used to raise antiserum in mice. Fig 2c shows that the pPF2L antiserum immunoprecipitates a 160kd antigen from *P.falciparum* (track 2).

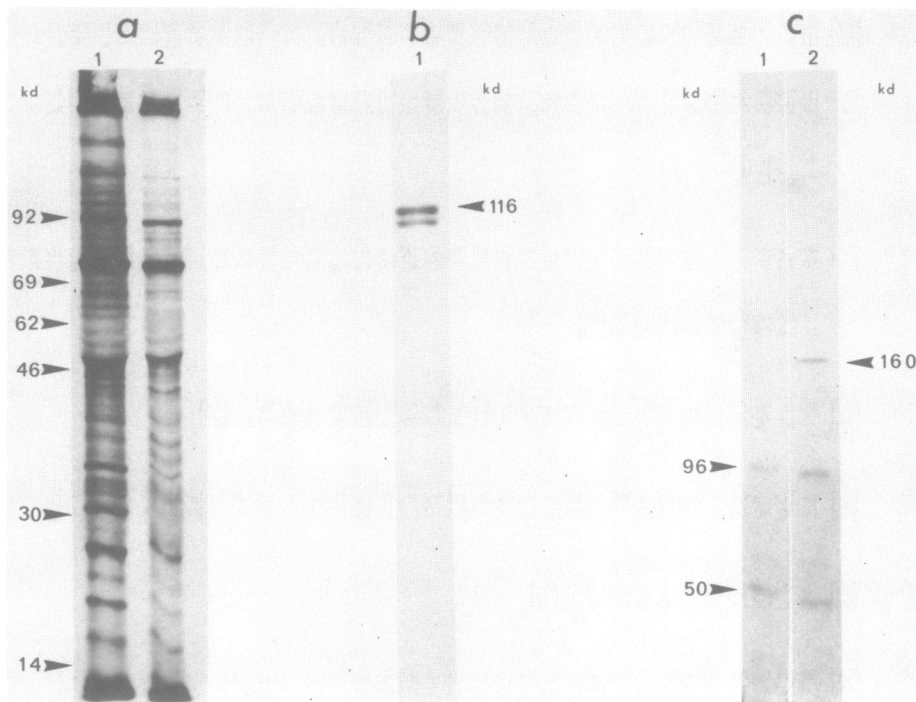


FIGURE 2 2a. Autoradiogram of S-methionine labelled erythrocytic stage proteins immunoprecipitated with protective monkey serum 422 (track 1) and non-protective monkey serum (track 2). The gel used contained 10% acrylamide. 2b. Autoradiogram of a Western transfer of the purified β -galactosidase fusion from pPF2L, probed with a pool of immune human sera. The second band results from a breakdown product of the chimera which is consistently generated during the purification process. Note that the fusion has approximately the same size (116kd) as β -galactosidase. If bacteria are harvested in log. phase, the fusion protein is degraded to a polypeptide of 80kd, that retains the antigenic determinant. 2c. Autoradiogram of parasite proteins recognised by non immune mouse sera (track,1) and sera from the same mouse, immunised with the purified fusion (track,2). Anti-pPF2L sera identifies a 160kd. blood stage antigen. The gels used in panels a and b contained 7.5% acrylamide.

The two proteins of 96kd and 50kd also immunoprecipitated by anti-pPF2L are due to non-specific binding, as they are also recognised by the pre-immune serum (track 1). Shown for comparison are the profiles of the protective and non-protective monkey sera (Fig2a) used to select this clone.

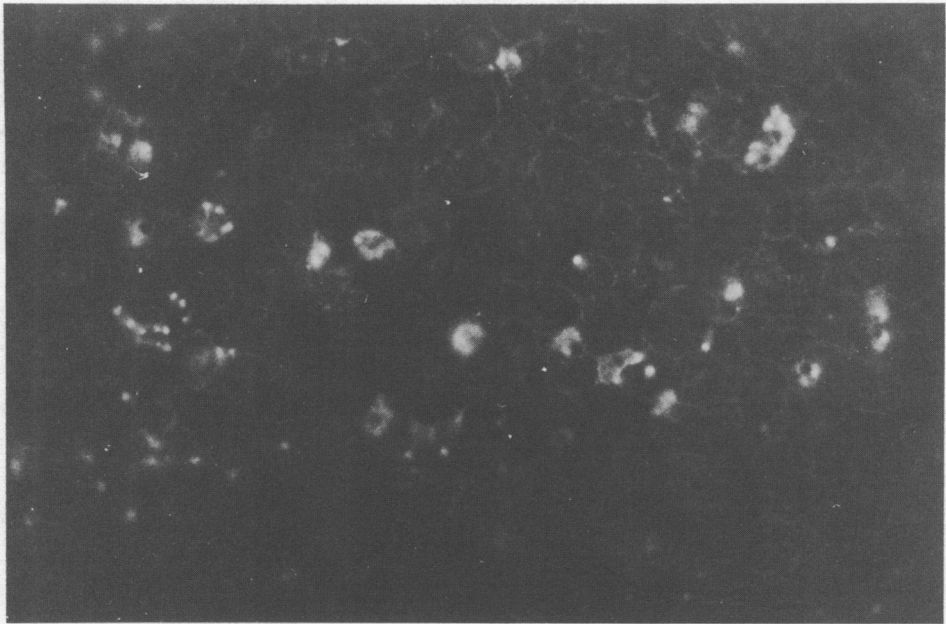


FIGURE 3 Indirect immunofluorescence of anti-pPF2L sera bound to parasite infected red blood cells. A diffuse pattern of fluorescence can be seen to all of the erythrocytic stages, including when present free merozoites. Note that there is no fluorescence from uninfected erythrocytes. Since the fluorescence pattern is diffuse and appears to cover the whole parasite we are unable to assign the pPF2L to a particular parasite structure.

The pPF2L antigen is present in all stages of the blood cycle

To identify at which stage of the erythrocytic development the 160kd. antigen is present in the parasite, indirect immuno-fluorescence (IFA) was used in a fixed parasite preparation (9). Figure 3 shows that the pPF2L antigen is located in all the blood stages, from rings to schizonts as well as merozoites. The pattern of fluorescence is diffuse and appears to cover the whole parasite, and as such we are unable to assign it to any of the classes proposed by McBride *et al.*, (18). Note that there is no reaction with uninfected erythrocytes, moreover unlike pPF11-1 and RESA there is no reaction with the membrane of infected erythrocytes (9, 19).

The pPF2L antigen is expressed in the blood stages and derived from a single gene

The 689bp insert of pPF2L was purified and hybridised to *P.falciparum* Palo Alto (FUP strain) genomic DNA. As shown in Figure 4a a single fragment is identified in each case. They are of 20kb (*Eco*RI, track 1), 25kb (*Hind* III, track 2) and 1.3kb (*Sau*3A, track 3). Figure 4b shows an autoradiograph of a Northern

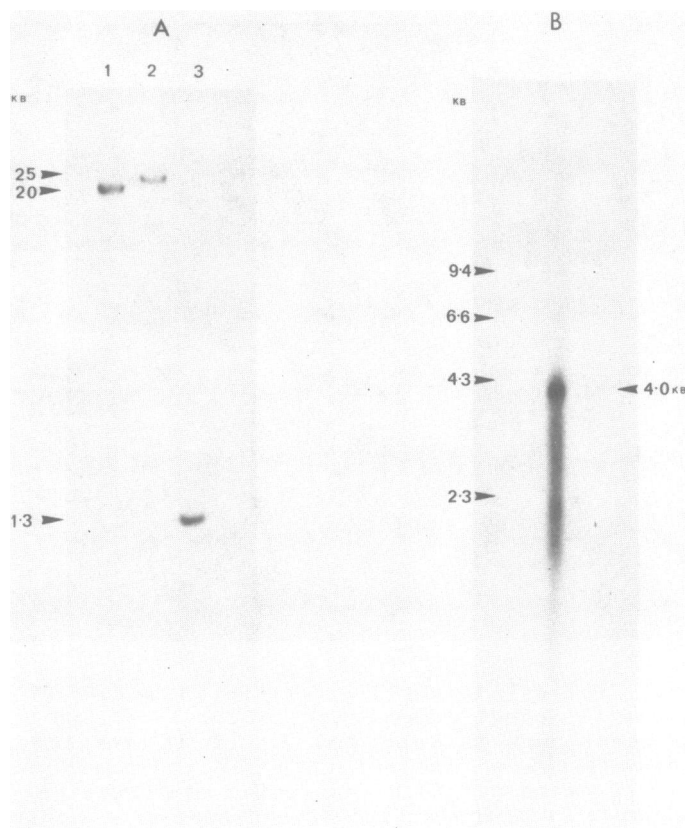


FIGURE 4A Autoradiogram of a Southern transfer. *P.falciparum* genomic DNA (2 ug. per track) was restricted, size fractionated on a 0.8 % agarose gel, transferred to nitrocellulose and then hybridized to the purified insert of pPF2L. Note that a single fragment is identified in each case. The restriction digests are, Track 1, EcoRI, Track 2, HindIII, Track 3, Sau3A. Sizes of fragments are given in kilobase pairs (kb.).

FIGURE 4B Autoradiogram of a Northern transfer. *P.falciparum* mRNA (2 ug.) was size fractionated on a 0.8% agarose/formaldehyde gel, transferred to nitrocellulose and then hybridized with the purified insert of pPF2L. rRNA and HindIII fragments of phage lambda were used as size markers. A single transcript of approximately 4kb. can be seen. Note that the mRNA appears to be degraded as there is a smear below the 4kb band. The primary transcript therefore might be slightly larger.

transfer. Here, a single mRNA with an apparent size of approximately 4kb can be observed. A transcript of this length is sufficient to encode a protein of 160kd. The observation that only a

FIGURE 5 The complete nucleotide sequence of the 689bp. of P.falciparum genomic DNA contained in pPF2L. Shown above the nucleotide sequence is the inferred amino-acid sequence of the pPF2L antigen. This corresponds to the open reading frame that is in frame with B-galactosidase, shown as the lac Z gene of the vector pUK270. The two other reading frames which are out of phase are shown underlined. Shown as well is the nucleotide sequence of the polylinker in pUK270, together with the length of the tails. The 4th and 6th codons of B_galactosidase are also indicated. The symbols for the nucleotides are : A, deoxyadenosine ; C, deoxycytidine; G, deoxyguanosine and T, deoxythymidine. The number of the base is given below the nucleotide sequence, where the first base is that of the P.falciparum insert.

single Sau3A fragment of 1.3kb is identified with the 689bp probe, taken together with the single HindIII and EcoRI fragments, leads to the conclusion that the pPF2L gene is probably unique. The identification of a transcript in blood stage mRNA is consistent with the IFA which showed that the erythrocytic stages present this antigen.

The nucleotide sequence and corresponding amino-acid sequence of the reading frame encoding the 160kd antigen are presented in Fig 5. The reading frame for the 160kd protein only commences at nucleotide 480 of the insert. The indication that the B-galactosidase fusion could be the result of a translation

reinitiation is consistent with the observation that the chimera does not have the expected increase in size due to an insert of 689bp coding for 229 amino acids (25kd), but is only slightly larger than β -galactosidase (Fig 2b); and moreover is produced only in limited amounts (0.5% of total *E.coli* protein compared to 10% for pPF11-1, data not shown). Its production in limited amounts may also explain the weak signals obtained in the primary and protective monkey sera screens.

There are two other open reading frames present on the 689bp, shown underlined in Fig 5. When sequence (0-460bp) that includes the two other open reading frames is used as a probe on a Northern blot of erythrocyte stage mRNA, no transcript is identified. This indicates that they are either not expressed, expressed at a level too low to be detected, or expressed in a different stage of the life-cycle. The same probe however, readily identifies unique bands on a Southern blot (data not shown). Finally the first open reading frame is out of phase with the promoter proximal amino-acid of β -galactosidase and is thus not translated in *E.coli*. We have no evidence that the second open reading frame, in this clone, is expressed in *E.coli*. The possible significance of the other reading frames is discussed.

DISCUSSION

The clones presented here represent a collection of *P.falciparum* epitopes. The data given in Table 1 demonstrate that the amount of antibody to these epitopes varies considerably in different immune sera, some are strongly recognised by immune human sera from Africa, whilst being apparently absent in an immune human serum from South America. (pPF11-1, pPF3-12). The reverse is also found (pPF20-3). Others respond equally well with the two human sera (pPF2L and pPF4-1). A differential response can also be observed between the protective and immune monkey sera (pPF3-12, pPF2L and pPF56-52). Whether these represent epitopes whose recognition is important in the development of protective immunity in the squirrel monkey model, remains to be seen.

The nucleotide sequence reveals that these small epitopes can be composed of unusual sequence. All but one are very A+T rich and clone pPF20-3 for example, encodes only 11 aminoacids of which 6 in a row are lysines. Small epitopes of unusual sequence may be a characteristic feature of *Plasmodia*, since the CS antigen has an epitope of only 4 amino acids (3, 4) and the histidine rich protein of *P.lophurae* has 9 histidine residues in a row (20). These clones need to be analysed further.

pPF2L was analysed in detail. It encodes an epitope carried on a 160kd antigen. The gene for this antigen is transcribed in the blood stages to yield a transcript of approximately 4kd. This is translated to give a protein, which by IFA is present in all of the erythrocytic forms. This data together with that we have previously published for pPF11-1 (9) encourages us to believe that all of these clones may represent genuine *P.falciparum* antigens.

The presence in pPF2L of other reading frames is an interesting feature. pPF2L may represent the 5' promoter region for the 160kd antigen gene. It does have a translational start (ATG) and upstream at position 427, there is a perfect TATAA box

(21). It does not appear to be an exon/ intron junction, as it lacks consensus splicing signals. Experiments are in progress to delineate these possibilities. Antisera to the large protein encoded by the large overlapping reading frame will also be raised, IFA will then indicate if this frame is expressed at a different stage of the parasites life-cycle. If true, this would demonstrate that *P.falciparum* can overlap certain genes. Apart from increasing the coding capacity of the genome, this would have implications for the stage specific regulation of gene-expression.

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